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# MEETINGS OF THE ROYAL BOTANICAL SOCIETY OF THE NETHERLANDS

## MEETING OF THE SECTION FOR THE RELATION BETWEEN PLANTS AND ANIMALS ON 19 NOVEMBER 1984

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### Bumblebees and constancy

During a ten years' study on the pollination of large-flowered Scrophulariaceae, numerous observations of bumblebee foraging were made. In this paper constancy is considered to include both flower constancy and constancy to a forage area. Bumblebees were individually marked for easy identification and followed during their foraging trips. Constancy could be demonstrated during a single foraging trip or during various trips on the same day or on different days. Bumblebees showed a high constancy to certain foraging areas. Large distances to new foraging sites (up to 1.8 km) were observed only if the preferred food plants were unavailable more closely. Some species, *e.g.*, *Bombus hortorum* and *B. pascuorum*, were spotted more often than other ones, such as *B. hypnorum* and *B. lapidarius*. Distances flown within a given area were never of any appreciable length. At low plant densities (not more than 98 *Rhinanthus* flowers/$m^2$) 45%, and at high densities (up to 473 flowers/$m^2$) 30% of the flight length did not exceed 10 cm, and only 10% of the flight lengths were over 40 cm at both densities.

*Rhinanthus serotinus* was the plant species preferred by most of the bumblebees except *Bombus lapidarius* and *B. pascuorum*, which showed a preference for *Trifolium pratense* and *Cirsium palustre*. Pollen-gathering individuals showed a lower constancy to *Rhinanthus* than nectar-collecting ones.

Microscopic analysis of pollen loads showed percentages of *Pedicularis* pollen of more than 80% if the bumblebees were captured on *Pedicularis*. Queens showed a somewhat lower purity of loads. Other plant species (often 2 or 3, more rarely 4) were normally represented in low percentages. Pollen loads collected later in the season showed a purity below 50% and contained up to 5 different plant species. This is due to a greater choice of pollen- and/or nectar-providing plant species. Mixed loads often consisted of layers of different colours, indicating that the bumblebees did not usually visit different plant species at the same time but remained faithful to a single species over a certain period of time. During a three-week period pollen loads of individual bumblebees showed that they remain faithful to one species or sometimes to the same two species.

Detailed analyses of the flower morphology of visited *Rhinanthus minor* and *R. serotinus* flowers, in conjunction with the expected pollen carry-over by the various bumblebee species enabled us to estimate the probable rate of hybridization between the two *Rhinanthus* species.

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### An underestimated and ancillary factor in the forage strategy of Bumblebees visiting Lupinus polyphyllus.

*Lupinus polyphyllus* is a protandrous pollen flower. In general, bumblebees forage on the inflorescences in an upward direction. They start at the base of the inflorescence where they visit older flowers to end at the top, visiting younger, fresh flowers.

This foraging behaviour is also found in the cases of *Digitalis* and *Aconitum*, whose inflorescences are of a similar architecture. Inflorescences of the latter two taxa show, in the upward direction, a decreasing nectar reward gradient, whereas the inflorescences of *L. polyphyllus* show, in the upward direction, an increasing pollen reward gradient.

The pollen of lupins is released in small amounts from the small aperture in the keel when the visiting bee presses the wings down, thus effecting pollination.

To effectively press down the floral wings the insect must have the right size and a strong enough power to overcome the stiffness of the wings. This stiffness can be considered to represent the turgescence of the wing tissues. In view of the differences in age of the flowers, it is to be expected that the younger flowers in the upper tiers have the stiffest wings, which, therefore, require the greatest force to be pressed down. The pattern of force distribution within the inflorescence most probably will show an increasing force gradient. This assumption was confirmed by means of Brantjes' torsion based force meter (range 0.1–0.3 dynes; BRANTJES 1981).

It may, accordingly, be supposed that because during visits an increasing effort will be required from the bee to gain access to the pollen, this leads to diminishing pollen returns. Our assumption is: the bee will end his visits and leave the inflorescence for another one, when the flower last visited does not present any reward. This is the case as soon as the bee does not succeed in overcoming the previously mentioned bio-mechanical force and, therefore, fails to collect pollen, or when, for all its (inadequate) efforts, the amount of pollen collected is disappointing.

If this hypothesis is correct it is in good agreement with the 'Optimal foraging theory' in the sense that the bees match their foraging behaviour to the actual and not to the potential reward gradient, and thus strive for a maximum rate of energy intake.

BRANTJES, N.B.M. (1981): Floral Mechanics in Phlomis (Lamiaceae). *Ann. Bot.* **47**: 279–282.

P.W. VROEGE *(Hugo de Vries-Laboratorium, Universiteit van Amsterdam, Plantage Middenlaan 2a, 1018 DD Amsterdam).*

Rate of dependence of two different Salix species on anemophilous and on entomophilous reproduction

It was tried to assess, by means of a simple experiment, the contribution of the wind and of insects as pollinators of *Salix repens* L. and of *S. caprea* L. The study was carried out in the dune area near Den Helder. About 40 female catkins were made inaccessible to insects by cageing them in, without hampering the access of air-borne pollen. In addition, *S. repens* was studied in two alto gether different locations, *viz.*, (l) a single female shrub exposed on all sides and surrounded by male individuals, and (2) a specimen in a N. -S. running gully surrounded by forest and with only a single male individual to the S. of it in the immediate vicinity. During the months of April and May, 1983, the weather conditions – temperature, wind-force, precipitation – were recorded in 244 six-hourly intervals.

The experiments with *S. repens* showed the following results: at the exposed site the contribution of insect pollination was 30%, in the for anemophily less favourable situation 80%. The percentages were calculated by counting very large numbers of seeds (at the first mentioned locality nearly 10,000). The pollination by insects must have taken place in a very efficient way, because in the inclement spring of 1983 the visiting could only take place in 23 six-hours' periods as against 107 deemed favourable for wind pollination.

In the *Salix caprea* plants studied insect pollination contributed to appr. 50% of the seed set.

The complete investigation report including a list of visiting insects, is available at request.

P.W. VROEGE *(Hugo de Vries-Laboratorium, Universiteit van Amsterdam, Plantage Middenlaan 2a, 1018 DD Amsterdam).*

Can predatory mites act as effective pollinators? A preliminary report.

During a study of the anthecology of *Calystegia soldanella* (L.) R. et Sch., *C. sepium* (L.) R. Br. and *Convolvulus arvensis* L. on a small, artificial peninsula in the Marsdiep near Den Helder, the behaviour of a predatory mite of the genus *Anystis* (Anystidae) drew my attention. This acarid was very numerous at the time: six individuals hiding in a single corolla tube were not exceptional. The rate of displacement of these mites is astonishing. The mites observed on the flowers behaved as possible pollinators: a fast run up a filament, a walk over the anther, down again and without stopping a climb to the stigma (or *vice versa*) and sooner or later a departure to a future destination.

For this reason a SEM study of a number of these carnivorous mites was undertaken. As might

be expected, the mites carried convolvulaceous pollen on their bodies, but also other pollen types such as that of liguliflorous composites. Since at the time the specimens of *Anystis* were caught all taxa with the latter pollen type were past flowering or had not been recorded from the peninsula, a process of elimination led to the conclusion that the pollen carried by the mites was from *Hieracium umbellatum* L., locally occurring in thousands. The plant of this species nearest to the place where the mites were caught on bindweed grew at a distance of about 35 metres and the animal carrying the pollen must have travelled a distance of at least 35,000 times its own bodylength. Accepting the suggested origin of the composite pollen, the mobility of this inconspicuous mite is amazing. Also pollen grains identified as *Artemisia* were present on the mite; both *A. maritima* L. and *A. vulgaris* L. occur on the peninsula.

S.C. WILLEMSTEIN *(Haagweg 266, 2324 NC Leiden)*

Evolutionary developments of the numbers of ovules per stigma in the Angiospermae

Among a number of statistical analyses of insect visits to flowers and of anthecologically important character states, one was devoted to the numbers of ovules per stigma (with some exceptions, in most taxa equivalent to the number of ovules per carpel). The analysis was based on records of flower visits by insects compiled in Knuth's 'Blütenbiologie' as far as it concerns the central European flora and the numbers of ovules per stigma recorded in Hegi's 'Illustrierte Flora von Mittel-Europa'.

In the analysis the minimum numbers of ovules are considered, because it was expected that an increasing specialization of the pollination process was accompanied by an increase of the number of ovules per stigma, and thereby indicating the maximum of established evolution. Correlation of the results of this analysis with the phylogeny and fossil record of the insect taxa in which anthophily developed showed that this is indeed the case in the evolution of entomophilous pollination: general entomophilous flowers or inflorescences (pollen becoming transported diffusely spread over, mainly, the ventral side of the body of the pollinators) significantly more often have one or few ovules per stigma than the more specialized melittophilous and psychophilous/phalaenophilous ones (pollen transport more concentrated on the body of the pollinators and thus more pollen grains becoming deposited on the stigma than in the case of the general entomophilous types).

Apart from adaptation of their floral morphology, some plants became better adapted to these specialized pollination types by shedding their pollen in the form of polyads or pollinia.

P.STELLEMAN *(Sportlaan 248, 3135 GZ Vlaardingen)*

Entomophily in mosses: A documented survey of the literature concerning spore dispersal of Splachnaceae by dung and carrion flies

Selected references:

BRYHN, N. (1897): Beobachtungen über das Ausstreuen der Sporen bei den Splachnaceen. *Biol. Centralbl.* **17**: 48–55.

CAMERON, R.G. & D. TROILO (1982): Fly-mediated spore dispersal in Splachnum ampullaceum (Musci). *Michigan Botanist* **21**: 59–65.

KOPONEN, A. & T. KOPONEN (1978): Evidence of entomophily in Splachnaceae (Bryophyta). *Bryoph. Bibl.* **13**: 569–577.

P. STOMMEN[1] and N.B.M. BRANTJES[2] ([1]Botanisch Laboratorium, Toernooiveld, 6525 ED Nijmegen; [2]1e Barendrechtseweg 37, 2991 XE Barendrecht)

Insect pollination in Hemp Agrimony, Eupatorium cannabinum L. (Asteraceae)

# MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE ON THE 8TH OF MARCH, 1985

## LECTURES

J. VAN AARTRIJK, G.J. BLOM-BARNHOORN and P. VAN DER LINDE *(Stichting Laboratorium voor Bloembollenonderzoek, Postbus 85, 2160 AB Lisse)*

### Dormancy and in vitro culture of lily

A.F. CROES, G.W.M. BARENDSE and G. VAN DEN ENDE *(Botanisch Laboratorium, Toernooiveld, 6525 ED Nijmegen)*

### Hormonal regulation of differentiation in vitro

Numerous differentiation processes, both at the biochemical and the morphological level, precede and accompany organ formation in callus or parenchyma *in vitro*. On tissue superficially cut from tobacco stems, flower buds, shoot buds and roots may be induced to develop directly, i.e. rapidly and without intervening callus phase. The system is, therefore, ideal to study hormone-directed differentiation. Development is regulated by two hormones: an auxin (α-naphthalene acetic acid) and a cytokinin (benzyladenine). Both exert an influence on a number of different facets of the developmental process. In general, the effects are specific for one hormone or the other. Only in a minor number of aspects such as maintenance of the tissue, the hormones interact.

Auxin and cytokinin are rapidly taken up from the medium. Despite an active conversion of both NAA and BA into polar metabolites, the concentrations of the free hormones in the tissue exceed those in the medium, especially in the case of NAA.

H.J. HUIZING *(Vakgroep Farmacochemie en Farmacognosie, Farmaceutische Laboratoria Rijks Universiteit, Ant. Deusinglaan 2, 9713 AW Groningen)*

### Manipulation of plant cells for production purposes

Generally, the initiation and establishment of *in vitro* cultures of plant cells has succeeded for many species.

In several cases, plant cell cultures have also been established with the view to study their plant cell technological application. On most occasions though, the derived plant cell cultures did not show a sufficient production of metabolites.

This observation has led to the application of media, especially designed for the enhancement of the productivities of the cells. Also physical factors have shown to be significant for the production of cells and metabolites. Only in a limited number of cases, information is available which describes the use of genetically transformed plant cells for production purposes.

Beside the possibility to use environmental factors for the manipulation of the autonomous production of plant cells, the application of biotransformation of exogenously supplied precursors by free or entrapped plant cells is considered nowadays for the production of both natural and synthetic compounds.

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### Induction of anthraquinone biosynthesis by biotic elictors in cell suspension cultures of Cinchona ledgeriana Moens

In previous studies (MULDER-KRIEGER et al. 1982, WIJNSMA et al. 1984) we reported on the isolation and identification of anthraquinones from callus cultures of *Cinchona ledgeriana*. Also in cell suspension cultures of the same species anthraquinones are formed. Although the genus *Cinchona* has been subject to extensive chemical studies, only one paper seems to have been published on a possible occurrence of anthraquinones (COVELLO et al. 1970). The reason for the occurrence of relatively

large amounts of anthraquinones in tissue cultures, whereas their occurrence in the intact plant seems questionable, might be that the callus tissue (in fact a sort of wounded tissue) forms anthraquinones for its protection again infections. Because the anthraquinones isolated from *C. ledgeriana* tissue cultures showed a clear antimicrobial activity (unpublished results), the hypothesis was developed that these anthraquinones act as phytoalexins. In the present study we report on the induction of the anthraquinone biosynthesis in cell suspension cultures of *C. ledgeriana* by addition of a sterilized mycelium filtrate of *Phytophthora cinnamomi*, a fungus known to be pathogenic to *Cinchona* species. Upon addition of the filtrate mentioned, to suspension cultures of *C. ledgeriana*, the anthraquinone content of the culture increased sevenfold, as compared with a non-treated culture. It seems, thus, that the anthraquinones mentioned may indeed act as phytoalexins.

MULDER-KRIEGER, TH., R. VERPOORTE, A. DE WATER, M. VAN GESSEL, B.C.J.A. VAN OEVEREN, & A. BAERHEIM SVENDSEN: Identification of the alkaloids and anthraquinones in Cinchona ledgeriana callus cultures. *Planta Med.* **46**, 19–24 (1982).

WIJNSMA, R., R. VERPOORTE, TH. MULDER-KRIEGER & A. BAERHEIM-SVENDSEN: Anthraquinones in callus cultures of Cinchona ledgeriana. *Phytochemistry* **23**, 2307–2311 (1984).

COVELLO, M., O. SCHETTINO, M.I. LA ROTONDE & P. FORGIONE: Riconoscimento e determinazione quantitativa dei derivati anthrachinonici di origine vegetale per via cromatografica. *Boll. Soc. Ital. Biol. Specim.* **46**, 500–503 (1970).

R. VAN DER HEIJDEN[1], R. WIJNSMA[1], R. VERPOORTE[1], P.A.A. HARKES[2] and A. BEARHEIM SVENDSEN[1] *([1]Vakgroep Farmacognosie, Gorlaeus Laboratoria, Postbus 9502, 2300 RA Leiden; [2]Vakgroep Moleculaire Plantkunde, Nonnensteeg 3, 3211 VJ Leiden)*

Production of indole alkaloids in tissue cultures of Tabernaemontana

The genus *Tabernaemontana* belongs to the Apocynaceae and comprises about 100 species distributed troughout the tropical as well as some subtropical parts of the world. Often its species are used in traditional medicine and for other purposes. The indole alkaloids present are probably responsible for their use.

Last year, after preliminary studies on the biological activity of some *Tabernaemontana* alkoloids (VAN BEEK 1984), a study on the biosynthesis and production of such alkaloids by means of tissue cultures was started. Meanwhile, callus cultures as well as some suspension cultures were initiated of about 10 species. Preliminary studies showed that alkaloids are produced in both callus and suspension cultures, although the composition of the alkaloid mixture and the amount of alkaloids may differ from that of the intact plant. Especially the alkaloids apparicine, tubotaiwine and vobasine were produced in tissue cultures of *Tabernaemontana* species. Also some dimeric indole alkaloids (such as monogagaine and 3-OH-conodurine) could be identified.

VAN BEEK, T.A.: *Pharmacological studies of some Tabernaemontana species.* Thesis, Leiden University, 1984.

A.M.M. DE LAAT *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

Genetic manipulation in plant breeding; first steps to chromosome transplantation

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Analytical and preparative aspects of electrofusion of plant protoplasts in a widely spaced electrode arrangement

The application of electric fields for aggregation and fusion of protoplasts has several interesting possibilities, i.e. a) analysis of fusion and uptake properties of protoplasts, b) study of early fusion and uptake events, c) rapid preparation of mechanically stable fusion products.

In a widely spaced electrode arrangement, the protoplasts can be made to align without contacting the electrodes (TEMPELAAR & JONES 1985a, b). When used on a small scale, analytical experiments

can be performed under the lightmicroscope, involving 200–400 protoplasts between the electrodes, and pulse duration- fusion response curves can be generated to quantify fusibility (TEMPELAAR & JONES 1985a). Apart from size, the origin turns out to be an important factor in this respect.

Preparative experiments can be done on the same scale as chemical fusion experiments, using millions of protoplasts. Early events in culture (wall formation, migration of chloroplasts, division) do not suggest ill effects of the electric field treatment and indeed callus and regenerating plants have been obtained (TEMPELAAR & JONES 1985a, DE VRIES et al. 1985).

TEMPELAAR, M.J. & M.G.K. JONES (1985a): Fusion characteristics of plant protoplasts in electric fields. *Planta* (in press).

TEMPELAAR, M.J. & M.G.K. JONES (1985b): Analytical and preparative electrofusion of plant protoplasts. *Oxford Surveys of Plant Molecular and Cell Biology* (in press).

DE VRIES, S.E., E. JACOBSEN, M.G.K. JONES, A.E.H.M. LOONEN, M.J. TEMPELAAR & J. WIJBRANDI: Somatic cell genetics of potato (Solanum tuberosum L.). III. Somatic hybridization of two amino acid analog-resistant cell lines by electrofusion. *Acta Bot. Neerl.* (this volume).

K.J. PUITE *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

Somatic hybridization of nitrate reductase-deficient mutants of Nicotiana plumbaginifolia by electrofusion of protoplasts

The technique of protoplast fusion in an electric field in order to obtain somatic hybrids (KOHN & SCHIEDER 1984) has been tested using two recessive nitrate reductase-deficient cell lines of *Nicotiana plumbaginifolia*: the CNX 20 line (NEGRUTIU et al. 1983), a cofactor mutant, and the NA 36 line (MARTON et al. 1982), which shows a deficiency in the apoenzyme part. In principle, somatic hybrids can be selected using culture media with nitrate as the sole nitrogen source (GLIMELIUS et al. 1978).

Mesophyll protoplasts of CNX 20 and fluorescein diacetate stained suspension protoplasts of NA 36 were exposed to electrofusion in a 150 µl chamber. The distance between the plate electrodes was 2 mm. After mutual dielectrophoresis of the protoplasts in an AC field of 150 V/cm at 1 MHz fusion was brought about by applying a DC pulse of 1.25–2 kV/cm during 50 µsec. The heterokaryons, which showed red chloroplasts fluorescence and yellow-green fluorescence due to fluorescein diacetate, could easily be recognized. The percentage of heterokaryons present was 3,5 – 4%.

After culture on a medium supplemented with casein hydrolysate cell colonies were washed and diluted with selective medium. A month after transfer to selective solid medium putative hybrids were collected. A number of these calli were tested for NR activity. They all appeared to be NR positive.

Control experiments using $\pm 10^5$ CNX 20 and NA 36 protoplasts in mono- and mixed cultures did nod lead to growth on selective media, while calli were grown on supplemented media.

GLIMELIUS, K., T. ERIKSSON, R. GRAFE & A.J. MULLER (1978): Somatic hybridization of nitrate reductase-deficient mutants of Nicotiana tabacum by protoplast fusion. *Physiol. Plant.* **44**: 273–277.

KOHN, H. & O. SCHIEDER: Somatic hybridization of two auxotrophic tobacco lines by electrofusion. *Abstract Int. Symp. Plant. Tissue and Cell Culture*, Olomouc, Sept. 1984.

MARTON L., T.M. DUNG, R.R. MENDEL & P. MALIGA (1982): Nitrate reductase deficient cell lines from haploid protoplast cultures of Nicotiana plumbaginifolia. *Mol. Gen. Genet.* **182**: 301–304.

NEGRUTIU I., R. DIRKS & M. JACOBS (1983): Regeneration of fully nitrate reductase-deficient mutants from protoplast culture of Nicotiana plumbaginifolia (Viviani). *Theor. Appl. Genet.* **66**: 341–347.

A.G.M. VOGELAAR *(Vakgroep Moleculaire Plantkunde MOLBAS, Biochemisch Laboratorium, Wassenaarseweg 64, 2333 AL Leiden)*

Introduction of kanamycine resistance in plants

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Development of methods for somatic cybridization of Solanaceae and the analysis of cybrids

A number of interesting plant genes are located in the cytoplasmic cell organelles. For instance cytoplasmic male sterility (CMS), a highly desirable trait for hybrid plant production, has been found to be encoded by mitochondrial DNA. In the tomato (*Lycopersicon esculentum*) CMS is not available. A possibility for the introduction of this trait into the tomato may be the transfer of cytoplasm from related Solanaceae.

In this paper we have shown the first step in somatic cybridization experiments between *Petunia hybrida* cytoplasts (enucleated protoplasts) with *L. peruvianum* protoplasts and we have described a method for analysis of cybrids.

This technique will be used for the transfer of cell organelles from different Solanaceous species (e.g. *Nicotiana tabacum, Solanum verrucosum* and *S. pennellii*) into the tomato, with the aim to introduce properties like CMS, as well as to study the segregation and/or recombination of cell organelles.

Before the character of CMS can be determined, flowering plants must be produced. A screening method as has been described, can be performed with small amounts of callus tissue, thus providing a preselection at an early stage for cybrids with the desired phenotype.

## POSTERS

J.H.W. BERGERVOET and J.B.M. CUSTERS *(Instituut voor de Veredeling van Tuinbouwgewassen, Postbus 16, 6700 AA Wageningen)*

Direct and indirect regeneration from cucumber (Cucumis sativus L.) explants.

In vitro regeneration of the cucumber is being attempted in our laboratory for several purposes, viz. obtaining somaclonal variation, facilitating chromosome elimination in callus originating from abortive embryos in interspecific crosses with the purposes to generate viable hybrid plants, and getting plants after fusion of protoplasts. Two different types of adventitious organ formation were discovered, direct regeneration of adventitious buds versus indirect regeneration of embryoids via callus. Direct regeneration of adventitious buds occurred, when big explants, e.g. 1–3 cm long hypocotyl sections, were incubated on MS medium with 3.5% sucrose, 0.05% trypton, 0.8% Difco Bacto agar, 50 μM K and 0.5 μM IAA in 24 h light. Indirect regeneration of adventitious embryoids via callus occurred, when small explants, e.g. 1–2 mm hypocotyl sections, were incubated on the same MS medium with 0.6% Difco Bacto agar, 4 μM BA and 4 μM 2,4-D in continuous darkness. Subculturing every two weeks was a prerequisite in the latter system.

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Isolation and regeneration of protoplasts from cucumber (Cucumis sativus L.)

Interspecific hybridizations between the cultivated cucumber and wild relative cucumber species are aimed at the introduction of disease resistances. Such hybridizations fail due to existing crossing barriers. We will investigate wether it is possible to overcome this problem by somatic hybridizations. A prerequisite is the ability to regenerate whole plants from isolated protoplasts.

Protoplasts were isolated from sterile cotyledons of 6 d old cucumber seedlings. The lower epidermis was removed by brushing and after preplasmolysis, the leaves were incubated for 16 h in a mixture of 1.5% cellulase R 10 and 0.3% macerozyme R 10 dissolved in a medium containing 0.25 M mannitol; 0.1 M glycine; 3 mM MES pH 5.6 and CPW(FREARSON et al. 1973). Protoplasts were released by washing and purified by filtration and centrifugation. The yield of viable protoplasts was $0.5 - 2 \times 10^6$ per gram tissue. Protoplasts were subsequently cultured in agarose containing medium: MS medium with 250 mg/l trypton, 0.25 M mannitol, 0.6% Sea Plague agarose, supplemented with 25 μM NAA and 15 μM 2iP. Routinely two plating procedures were used. Protoplasts

were mixed with the agarose medium and plated in thin layers (1.5 ml per 6 cm petri dish) and after 14 days segments of this layer were cultured in an agarose bead type culture according to SHILLITO et al. (1983). Alternatively the same protoplast suspension in agarose-medium was plated in 100 µl droplets (5 droplets per 9 cm petri dish). After gelling, 4 ml of liquid medium of the same composition was added and this agarose disc culture was incubated on a rotary shaker (30 rpm). For some protoplast preparations cell division and callus growth was improved using this last procedure, for other preparations both methods were satisfactory. If protoplasts were plated at a rather high density ($10^5$ per ml) a very high plating efficiency (80%) was obtained. However, lowering the plating density resulted in rapid decrease in plating efficiency.

The osmotic value of the medium was gradually lowered and small microcalli were isolated about 4–6 weeks after plating and were subcultured on agar media. Investigations are now directed towards the induction of shoots by changing the phytohormone ratio.

FREARSON E.M., J.B. POWER & E.C. COCKING (1973): The isolation, culture and regeneration of Petunia leaf protoplasts. *Developmental Biology* **33**, 130–137.

SHILLITO, R.D., J. PASZKOWSKI & I. POTRYKUS (1983): Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. *Plant Cell Reports* **2**, 244–247.

A. CALLEBAUT *(Instituut voor Scheikundig Onderzoek, Museumlaan 5, 1980 Tervuren, België)*

## Cell suspension cultures as a tool in studying active defense mechanisms in Cucumis sativus L.

Upon infection, cucumber plants respond by several active defense mechanisms: accumulation of phytoalexines, lignin deposition and increase of hydroxyprolin rich glycoproteins in the cell walls.

We induced cell suspension cultures of *Cucumis sativus* L. cv Marketer and infected them with spores of the fungus *Cladosporium cucumerinum*. Some of these interactions resulted in the accumulation of fungitoxic compounds in the cultured cells, as detected by bioassay. These fungitoxic products are the same as those found previously in infected plants and callus tissue. The variability of the induction of fungitoxic products can possibly be explained by the variation in growth cycle of the cells at infection. Cell walls of infected cells in suspension cultures clearly show an increase in lignin-like material, as shown by phloroglucinol and toluidine blue staining. In control cultures, small amounts of lignin deposition are also detectable, depending on the growth stage.

Cucumber cell suspension cultures seem to be a good model system for investigation of lignification and induction of fungitoxic products as disease resistance mechanisms.

L.J.W. GILISSEN and M.J. VAN STAVEREN *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

## Zinc-resistant cell lines of Haplopappus gracilis

Genetic markers with expression at the cellular level are useful tools in plant cell physiology and in the development of somatic cell genetics and chromosome transplantation. For the latter, *Haplopappus gracilis* (Nutt) Gray was used as a model species because of its low number of chromosomes (2n = 4). Zinc-resistance was choosen for as a possible marker character.

Zinc ions, when present in solid or liquid Gamborg B5-medium in a concentration of 7 mM or higher, were completely toxic to wild type cells of *H. gracilis*. Several selection procedures (at zinc concentrations between 7 and 20 mM) ultimately resulted in three cell lines able to grow well on 7 mM zinc.

The depletion of zinc from the medium was equal for the zinc-resistant and the wild type cell lines, when cultured in medium containing a non-toxic zinc concentration of 2.1 mM. MATHYS (*Physiol. Plant.* **40**: 130–136, 1977) suggested that in a number of higher plants an increase of cellular malate might result in zinc-resistance by shuttling the zinc ions through the cytoplasm into the vacuole, where they will be bound to terminal acceptors, like oxalate. However, gaschromatography showed equal amounts of malate and absence of oxalate in the selected and in the wild type cell lines of *H. gracilis*.

It is concluded that zinc-resistance in the selected cell lines of *H. gracilis* was caused neither by

exclusion of zinc ions, nor by increased endogenous content of malate.

Further cell genetic research is in progress to prove the usefulness of the selected zinc-resistant cell lines as genetic markers.

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Analysis and identification of metabolites in plant cell biotechnology by GLC and GLC-MS

Results already published in:
*Analytica Chimica Acta* (1984) **163**: 43–54.

Ch.H. HÄNISCH TEN CATE and B. DE GROOT *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

Morphology and characteristics of tumor calli from potato

M.HEIDEVELD, A.L. DE MAAT and A.J. KOOL *(Vakgroep Moleculaire Genetica Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam)*

Stimulation of growth of cell suspension cultures of Lycopersicum esculentum cv. Bellina by phosphate addition

A. HEMRIKA-WAGNER *(Biologisch Laboratorium Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam)*

Relation between pentose phosphate pathway mediated respiration and alternative pathway in callus forming potato tuber tissue

H.J. HUIZING, J. HIBMA and H.J. WICHERS *(Vakgroep Farmacochemie en Farmacognosie, Farmaceutische Laboratoria, Rijks Universiteit Groningen, Ant. Deusinglaan 2, 9713 AW Groningen*

Investigation on the feasability of crown-gall tumour tissue of Mucuna pruriens for production of L-DOPA

L-DOPA (dihydroxy-phenyl-L-alanine) is used for the symptomatic relief of Parkinson's disease. *In vitro* grown cells of *Mucuna pruriens* are able to synthesize this amino-acid. Up to now an amount of maximally 9% of L-DOPA, compared to the dry weight of the cells, has been found in cell suspension cultures. Generally, 0.5–2.0% is produced endogenously in the established cell suspension cultures.

Because of our interest in plant growth regulator (PGR) autotrophic cell lines, crown galls were initiated *in situ* on *Mucuna pruriens* plants by means of the *Agrobacterium tumefaciens* strain 4001. The formed tumours were isolated and transferted to Murashige and Skoog medium. PGR's were omitted. Bacterial cells in the tissues were killed by a number of weekly transfers of the calluses to media containing antibiotics. One bacterium-free cell line showed lysopine dehydrogenase activity, which was an indication, in addition to the observed PGR autotrophic growth, that these cells had been transformed with T-DNA.

It appeared that the transformed cells did not produce more L-DOPA than did cells which were grown in the presence of PGR's.

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Aspects of the application of in vitro (DNA) transformation systems in plants

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**The essential oils from in vitro grown shoots and from callus of Rosmarinus officinalis L. var. genuina forma erectus**

Studies on the formation and composition of essential oils produced by tissue cultures of essential oil bearing plants are scarce. However, results obtained so far show that the production of secondary metabolites by tissue cultures differs from that by intact plants. The main goal of the present investigation was to study the possibility of production of essential oil by *in vitro* cultures of proliferating shoots of rosemary, and to compare the composition of the oil thus obtained with that isolated from field grown plants.

Shoot apices of the variety of rosemary mentioned above were established in aseptic culture on a modified White's medium and proliferated on a modified Murashige and Skoog's medium, while callus was grown on a modified Schenk and Hildebrandt's medium.

The essential oil content of the *in vitro* grown shoots was in increasing order in 20, 30 and 40 days-old shoots; after 40 days the content was 1.8%. Shoots of 1 year-old field grown plants contained 2.4% essential oil, determined by solvent extraction. The essential oil content in callus was 0.42%, determined by the same method. Whereas by solvent extraction 1.8% of essential oil was found in 40 days-old *in vitro* shoots, only 1.2% was found when the oil was isolated by hydrodistillation using a Clevenger-type apparatus.

Results of the analysis by capillary GLC of the various oil samples – produced by *in vitro* culture of shoots, by callus and by field grown plants – will be presented.

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**Phytoecdysones in Ajuga reptans plants, callus and cell suspension cultures**

Phytoecdysones are ecdysteroids with insect-molting activity found in plants. It has been suggested that they play a role in the defense of plants from insect attack.

Ecdysterone and cyasterone were isolated from *Ajuga remota*, an East African medicinal plant. We investigated the ecdysterone content of plants, callus and cell suspension cultures of *A. reptans*, a common species in Western Europe.

Ecdysterone is identified in *A. reptans* plants by TLC and HPLC. In calli cultured on Murashige-Skoog medium with 2,4-D as auxin, traces of ecdysterone are present. Ecdysterone is not present in calli cultures on MS with NAA as auxin.

The quantitative analysis is hindered by an unidentified product, present in calli but not in plants, running close to ecdysterone on TLC and HPLC.

Ecdysterone could not be detected in cell suspension cultures, where again the unknown product is present.

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**Polycentric and mega chromosomes in cell suspensions of a dihaploid potato.**

The chromosome complements in three cell suspension lines of an aminoethyl-cysteine resistant cell variant of an interdihaploid ($2n = 2x = 24$) *Solanum tuberosum*, genotype $H^2578$, were investigated by using Giemsa-staining, C-banding, or differential staining after bromodeoxycytidine incorporation. Although the suspensions originated from calluses of various ages and had been in culture for different times, the complements had changed to the same degree.

The chromosome numbers of 60 metaphases varied from 33 to 151, though mainly between 36 and 79. 147 polycentric chromosomes, of which 142 dicentrics, occurred in 54 metaphases. The number per metaphase varied from 0 to 8, independent of the chromosome number. The frequency was 3.7 per 100 chromosomes. Fragments were found in 19 metaphases. The number varied from

0 to 5, and was not correlated with the number of polycentrics. The frequency was 0.7 per 100 chromosomes. 38 out of 60 anaphases showed 1 – 3 bridges.

Allopolycentrics (distal arms differing in length) occurred 3.4 times more frequently than isopolycentrics. The variation in the numbers of polycentrics was mainly due to the allopolycentrics. If the polycentrics were present in multiple per metaphase, they differed in morphology. 101 differentially stained polycentrics showed the normal staining pattern and one isodicentric had a switch in the differential staining in the middle of the intercentric segment. This switch might have been caused by a sister chromatid exchange or by an asymmetrical reciprocal translocation. Polycentrics apparently survive through parallel division of heterocentricity; nondisjunction is not involved.

Megachromosomes, which are subtelocentric chromosomes 4 to 13 times longer than the longest potato chromosome, were found in $\ll 1‰$ of the metaphases and never more than one per metaphase. They did not contain additional heterochromatin and apparently arise through a fusion between an extremely long monocentric fragment of a broken dicentric and a telomeric fragment.

L.H.W. VAN DER PLAS and B. OTTO *(Biologisch Laboratorium Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam)*

**Possible relationship between the respiratory metabolism of cell cultures of Petunia hybrida and cytoplasmatic male sterility**

Cytoplasmatic male sterile plants are not able to produce fertile pollen. The genetic information for this property probably is localized in the mitochondria. Cell suspensions were derived from male sterile en fertile *Petunia hybrida* plants and also from originally sterile *Petunia* plants, in which the fertility is restored. The respiratory metabolism of these cells was studied during cultivation in batch culture.

The capacity of the CN-resistant, alternative electron transport pathway was higher in the cells from the sterile plants. The quotient of the capacity of this alternative pathway and of the cytochrome pathway was higher in cells from sterile plants during cultivation at 16 °C, but not during cultivation at 28 °C. A decrease of the growth temperature from 28 °C to 16 °C caused an increased capacity of the cytochrome pathway in cells from fertile and restored plants, but this capacity remained unchanged in cells from sterile plants.

The amount of dry weight produced during growth in batch culture with a fixed amount of sugar was highest in cells from fertile plants and lowest in cells from sterile plants. However, the amount of ATP produced (calculated from the respiratory data) was highest in cells from sterile plants and lowest in cells from fertile plants. So, the dry weight production per mol ATP was much lower in cells from sterile plants. These cells seem to grow less economically and "spill" a part of the ATP produced.

Perhaps, this less efficient usage of ATP in the cells from the sterile plants is connected in some way with the incapacity to produce fertile pollen.

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**A morphometric analysis of cellular changes during in vitro germination of immature embryos from Zea mays L.**

M.M.C. TAN, F. VAN DER MARK and A.J. KOOL *(Vakgroep Moleculaire Genetica, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam)*

**Development of methods for somatic cybridization of Solanaceae and analysis of cybrids**

H.A. VERHOEVEN *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

**Synchronization, flow cytometric sorting and micro injection studies using Nicotiana plumbaginifolia cells and protoplasts**

H. VAN WEZEL, G. VAN DEN ENDE and A. CROES *(Botanisch Laboratorium, Toernooiveld, 6525 ED Nijmegen)*

## Change in capacity to regenerate buds in vitro on thin-layer tissues of tobacco

Tissue explants cut from the floral ramifications of tobacco (*Nicotiana tabacum* L. cv. Samsun) are capable to form buds in tissue culture.

Explants from recently formed internodes and flower stalks produce more buds than tissues cut from older parts of the ramification. This gradient in regenerative capacity mainly depends on the age of the tissue at the moment it is taken from the plant. The question is what brings about this change in capacity of the explants to form buds.

To study this problem, explants from young internodes and flower stalks were first incubated for 7 days in non-inductive conditions and then transferred to a medium inducing bud formation. Those tissues regenerate considerably more buds than tissues of comparable age left in place on the plant.

It is concluded that the *in vivo* decline in morphogenetic competence can at least be delayed experimentally. This suggests that in the plant factors from outside the tissue itself lead to physiological modifications which manifest themselves in a reduced regenerative capacity.

E.J. WOLTERING *(Sprenger Instituut, Haagsteeg 6, Postbus 17, 6700 AA Wageningen)*

## Ethylene and carbon dioxide accumulation within various tissue culture systems

Ethylene and carbon dioxide accumulation were determined within polypropylene tissue culture systems, containing *Gerbera jamesonii* cv. Sue Ellen plantlets (Vitroflora Ltd. Wagenigen) on rooting medium.

A comparison was made between a closed (sealed) system, a closed system with an ethylene absorbant ("Ethysorb"), an "open" (not sealed) system (used in commercial practice, resistance coefficient (R) = $2 \times 10^6$ s/m) and a system with a gas diffusable filter mounted in the lid (R = $4 \times 10^4$ s/m).

Within 26 days, ethylene and carbon dioxide concentrations increased to respectively 1.25 ppm and 13% in the closed system; 0.001 ppm and 10.5% in the closed system with 'Ethysorb"; 0.15 ppm and 1.5% in the "open" system and to 0.02 ppm and 0.2% in the system with filter.

Chlorophyll content of the leaves was highest when plants were grown in the system with filter, followed by plants from the open system, plants from the system with "Ethysorb' and plants from the closed system.

Beside ethylene and carbon dioxide some other gaseous components (emitted by the polypropylene material) were also detected in the atmosphere of the tissue culture system. Nevertheless, the experiments indicate that both elevated ethylene and carbon dioxide concentrations can have detrimental effects on growth and development of plants grown in tissue culture systems.

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## Somatic cell genetics of potato (Solanum tuberosum L.). I. Production of monohaploid lines and selection for tissue culture qualities

In a joint project of the Department of Plant Breeding (Agricultural University, Wageningen), the Research Institute ITAL (Wageningen) and the Department of Genetics of the University of Groningen, financed by the Foundation for Technical Sciences (STW), research is going on to construct a gene map of potato to pave the way for genetic engineering of that crop.

Via prickle-pollination (pseudogamy) and anther culture, more than 100 monohaploid lines of different *Solanum* species and interspecific hybrids have been produced. These single genome lines (12 chromosomes) are screened for stability of ploidy level after several cycles of shoot multiplication and growth *in vitro*. Ten different lines from four different genetic sources could be selected up to now. These lines were then screened for the ability to produce callus and to regenerate plants after protoplast isolation and fusion. At this stage of research we are able to produce calli out of protoplasts from most of the genotypes and regenerate plants from one of them. By way of

PEG ($Ca^{2+}$, high pH) method, fusion frequencies of more than 5% have been realized. We have some calli growing after a fusion treatment though it is not yet known whether these are hybrids or not.

Another aim is to produce from each monohaploid a series of homozygous clones with the ploidy levels x, 2x, 3x and 4x in order to study gene dose effects. At this moment x-2x-4x series of six monohaploids have been obtained. They are being investigated for vigour and fertility.

H.M.J. PENNINGS *(Stichting ITAL, Postbus 48, 6700 AA Wageningen)*

Somatic cell genetics of potato (Solanum tuberosum L.). II. Isolation and characterization of variant cell lines

In order to develop a genetic map of the potato there is a need for mutants, which can serve as genetic markers *in vitro*.

Attention was focussed on the induction, selection and characterization of fermentation mutants. In order to isolate this type of mutants, cell suspension cultures of the dihaploid line HH260 were mutagenized with chemical mutagens or X-ray irradiation. Whereas the use of N-nitroso-N-methylurea or N-methyl-N-Nitro-N-nitrosoguanidin did not result in an increase of mutant frequency, X-ray irradiaton (20–40 Gy) resulted in a small increase (2–3 fold) of galactose-utilizing variants. However, spontaneous mutant frequency was sufficiently high to isolate mutants.

About 100 variants, capable of utilizing galactose or mannose as the sole C-source, were isolated and characterized. In contrast to the wild type cells galactose or mannose is taken up by the variant cell lines and is metabolized. No loss of the variant character was observed when the cell lines were cultured under selective as well as under non-selective conditions. To verify the mutant nature of these variants hybridizations of these variants with wild type potato (2n) protoplasts will be carried out. For purposes of somatic cell genetics the chromosome loss and concomitant loss of character will be studied after fusion with *Nicotiana plumbaginifolia*.

In the near future attempts will be made to isolate mutants after transformation of leaf explants with *Agrobacterium tumefaciens* and T-DNA mediated transfer of the *neo* gene.

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Somatic cell genetics of potato (Solanum tuberosum L.). III. Somatic hybridization of two amino acid analog-resistant cell lines by electrofusion

Cell lines resistant to the amino acid analogues S (2-aminoethyl)-L-cysteine (AEC) or to 5-methyltryptophan (5 MT) have been isolated in the dihaploid potato clone HH 578 (JACOBSEN et al. 1985; JACOBSEN, unpublished results).

It has been examined whether some of these variants could be used as genetic markers in somatic hybridization experiments. Two cell lines appeared to be very suitable fusion partners. The variant 5 mt-26, which is resistant to 5 μM 5 MT and cross resistant to 100 μM 3-fluorotyrosine (3 FT) proved to be very sensitive to AEC (100 μM). The growth of aec-1, a variant resistant to at least 100 μM AEC, was completely inhibited when 5 μM 5 MT or 100 μM 3 FT had been added to the culture medium.

Cell suspension protoplasts of aec-1 and 5 mt-26 were fused in an electric field following the procedure described by TEMPELAAR & JONES (1985). The percentage of fusion varied between 10 and 20. During the first three months of culture the protoplasts were grown under non-selective conditions. By now small calluses have been transferred to non-selective solid culture medium. After further propagation selection will be carried out by growing small pieces of callus on culture medium to which both AEC and 5 MT or AEC and 3 FT have been added.

JACOBSEN, E., R.G.F. VISSER & J. WIJBRANDI (1985): Phenylalanine and tyrosine accumulating cell lines of a dihaploid potato selected by resistance to 5-methyltryptophan. *Plant Cell Reports* (accepted).

TEMPELAAR, M.J. & M.G.K. JONES (1985): Fusion characteristics of plant protoplasts in electric fields. *Planta* (in press).

## MEETING OF THE SECTION FOR WILD FLORA PROTECTION ON 10 OCTOBER 1984

E. ARNOLDS *(Biologisch Station Landbouwhogeschool, Kampsweg 27, 9418 PD Wijster)*

### The changing mycoflora of The Netherlands: facts and backgrounds

Changes in the flora of macrofungi are difficult to establish because macrofungi can only be identified by means of carpophores. These carpophores show a strong periodicity and great annual fluctuations, caused by variation in weather conditions. Besides taxonomic confusion is considerable and the knowledge on the mycoflora before 1950 is only fragmentary.

However, some methods of investigation proved to be successful: (1) The comparison of sets of 15 complete excursion lists from well-known areas, studied in the periods 1912–1954 and 1973–1982; (2) Distribution maps of some well-known species in different periods; (3) The results of mycocoenological permanent plots.

All three methods reveal similar tendencies. Generally spoken lignicolous fungi have increased during the latest decades, in particular species with a (facultative) parasitic way of life, e.g. *Fomes fomentarius* and *Panellus serotinus*. A recently established and increasing wood-saprophyte is *Pycnoporus cinnabarinus*, recorded since 1967. Among the litter-saprophytes most species are approximately constant. Some species increased, generally fungi from rich soils and man-made habitats, e.g. *Stropharia aurantiaca*. In the contrary some species from oligotrophic habitats decreased, e.g. *Cantharellula umbonata* and *Coltricia perennis*. Most fungi from grasslands declined significantly due to the increased use of artificial fertilizers, e.g. *Camarophyllus niveus* and *Hygrocybe psittacina*.

However the most striking changes concern species forming ectomycorrhizal associations with trees. The majority shows a statistically significant decline, in particular species of the genera *Cortinarius, Suillus, Cantharellus, Gomphidius* and *Tricholoma*, as well as all hydnaceous fungi. Some species which were formerly widespread and common have become extremely rare, e.g. *Dermocybe cinnabarina* and *Sarcodon imbricatus*. Quite a number even seems to have become extinct in the Netherlands, e.g. *Craterellus cornucopioides, Hygrophorus russula, Hydnellum aurantiacum, H. caeruleum, Phellodon confluens, Bankera fuligineoalba* and *Sistotrema confluens*. Only a few species of mycorrhizal fungi show a marked increase, e.g. *Lactarius hepaticus* and *Russula ochroleuca*.

The general decline of mycorrhizal fungi cannot be ascribed to changes in forestry, lowering of the groundwater table, climatological fluctuations or the influence of collectors. Strong indications exist that the decrease is caused directly or indirectly by air pollution, known as "acid precipitation". The changes in the distribution patterns are correlated with the spatial pattern of $SO_2$ concentration in the Netherlands. At present many declining species are restricted to calcareous soils with a large buffer capacity, whereas they were widespread about 1950.

ARNOLDS, E. (Ed.). *Veranderingen in de Nederlandse mycoflora*. Wetensch. Meded. K.N.N.V. no. 167, 1985.

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### Decline of epiphytic lichens in The Netherlands

A detailed picture of the decline of epiphytic lichens in The Nederlands can be made by comparing the Rijksherbarium's holdings of material from ca. 1850–1900 with the present situation. To do this, three strategies are possible:

1. comparison of the past and present flora of The Netherlands
2. comparison of past and present distribution areas of species
3. comparison of past and present vegetation types.

These strategies will be discussed in some detail below.

Ad 1. A major problem is the absence of a checklist of Dutch lichens, apart from the very incomplete list in the *Prodromus Florae Batavae* (1898). Furthermore, most of the Rijksherbarium's material has not been revised in recent times and many of the old identifications are unreliable. As an example, data are presented for the Caliciaceae, which have recently been revised by L. Tibell. Before 1910,

15 species were recorded; after 1970, eight of them were not found again, but 4 new species were recorded. The disappeared species occurred on wood (4), twigs (1) or were parasites; of the newly found ones, 3 occurred on bark and one was a parasite. Some species commonly occurring on wood in the past are now only found on bark. No strong decline of the Caliciacean flora becomes apparent in this way, but it should be noted that (a) this method is insensitive to species abundance and (b) in recent times sampling has been much more intensive than in the past. Species that are now confined only to a few localities may have been very common in the past.

Ad 2. Most of the older Dutch lichenologists paid little attention to microlichens. Therefore, a reliable comparison of distribution areas is only possible for the more conspicuous species. The distribution of a common species (*Parmelia acetabulum*) shows that sampling was fairly evenly distributed over the country with the exception of a "gap" in the province of Limburg. A comparison with the present situation shows that many species that formerly occurred all over the country are now confined to (a) the northern part of the country (e.g., *Physconia pulverulenta*), (b) the coastal area (e.g., *Physcia aipolia*) or (c) some "old forest" relics (e.g. *Usnea* spp.). Other examples are given by BARKMAN (1958) and DE WIT (1976).

Ad 3. Since pieces of bark constitute the samples they usually contain more then one species. Reconstruction of vegetation types is sometimes possible with the aid of these "accompanying" species. This was done with material collected around the turn of the century by J. H. Wakker in the surroundings of 's-Hertogenbosch (province of Brabant) (VAN DOBBEN 1983). When the area was re-sampled in 1973, only 46 of the 115 species found by Wakker could be traced. The diversity in vegetation types had also been reduced drastically. A comparison of past and present species lists, vegetation types and their substrates shows that species that are now extinct were formerly confined to only one vegetation type or one substrate; species with a wider amplitude usually still occur in the area. Furthermore, species formerly occurring on both acid and neutral substrates are now often confined to neutral bark. These phenomena may be explained from air pollution and resulting substrate acidification. A few species have expanded; these are either strongly acidiphytic species (e.g., *Lecanora conizaeoides*), or strongly nitrophytic ones (e.g., *Xanthoria polycarpa*).
Results from field and herbarium studies may be summarized as follows:

1. there has been a strong decline of epiphytic lichens over the past century;
2. decline has been most severe for species with a narrow ecological amplitude which were confined to e.g. decorticated wood, twigs, very old trees or old forests;
3. decline has been most severe in the southern part of the country and shows a strong spatial correlation with $SO_2$ levels;
4. there has been no or little decline for some common species with a wide ecological amplitude, e.g. *Parmelia sulcata, Evernia prunastri*;
5. some acidiphytic species have expanded: *Lecanora conizaeoides, L. expallens, L. saligna, Buellia punctata, Chaenotheca ferruginea*;
6. some nitrophytic species may have expanded too, especially over the past decade: *Physcia dubia, Ph. stellaris*;
7. the decline is almost completely due to air pollution; other factors (drought, habitat destruction) play a minor role;
8. areas with decreasing $SO_2$ levels show a re-invasion of species over the past decade.

BARKMAN, J.J. (1958): *Phytosociology and ecology of cryptogamic epiphytes.* Van Gorcum, Assen
VAN DOBBEN, H.F. (1983): Changes in the epiphytic lichen flora and vegetation in the surroundings of 's-Hertogenbosch (The Netherlands) since 1900. *Nova Hedwigia* 37, 691–719
DE WIT, A. (1976): *Epiphytic lichens and air pollution in The Netherlands.* Bibl. Lichenol. 5. Cramer, Vaduz

54

W. RUBERS *(Joh. Frisoplaats 6a, Tricht)*

## Distribution maps of Netherlands bryophytes

From the distribution maps the general impression is that bryophytes show a similar degree of decline as phanerogams do. It is estimated that 82 mosses (excluding *Sphagnum* and Hepatics) 25% of our contemporary flora, form a category of seriously endangered species. Losses are most severe in wet habitats, in particular ombrotrophic bogs, marshy meadows, dune slacks and wet heathlands. Together with a decline, a number of species show an apparent shift in ecological preference. Separate distribution maps of their respective habitats made some striking examples visible. Separate maps of capsule bearing plants show for a number of species an alarming reduction of fertility. Though scientific proof is lacking there is no doubt that the worsening composition of the rain will turn out to be the injuring cause.

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## Threats to and conservation of bryophyte communities and habitats in The Netherlands

The large number of bryophyte species and the high diversity of bryophyte communities formerly found in The Netherlands is for a large part due to the old, consistent pattern of human activities. Changes in this pattern during the last century are mainly responsible for the considerable recent impoverishments. This includes the large-scale problems of air and water pollution, eutrophication of water, soil and rocks, and use of herbicides. The old small-scale pattern of land use with high local constancy has changed into a large-scale pattern with much more temporal variation on each site, resulting in loss of suitable habitats – most coppice woods are turned into meadows or forests, earth walls with hedges are removed or their maintenance is neglected, small loam-, clay- and chalk-pits are no longer in use, car tracks in the dunes are abandoned, *etcetera*.

Even in nature reserves, bryophyte communities may be lost or become impoverished, partly because changes in management regimes took place for economic or other reasons (e.g., mowing of chalk grassland in stead of the old practice of sheep grazing).

Bryophytes are especially vulnerable because they do not possess roots and remain of low stature; so, they are not able to profit from buffering capacities of the substrate. Also, the "life strategy spectrum" of bryophytes as a group is different from that of phanerogams: perennial species with potentially very long-lived individuals are much rarer and short-lived shuttle species or colonists are more common among bryophytes. This is related to the more temporary nature of many bryophyte habitats, and the lower competitive ability of bryophytes as compared to phanerogams. So, dispersal to new sites is of greater importance for bryophytes, in general. This means that a reasonably high density of populated sites is important for maintenance of community diversity and for survival of the species, even though those species that produce spores are thought to disperse rather easily – most species depend on vegetative reproduction or have large spores, and both methods of propagation are not suitable for long-distance dispersal.

Protection of bryophytes in the first place means a reduction of the level of air and water pollution and of the general lowering of the water table. Also, maintenance of many small landscape elements such as earth walls, steep road sides, small loam-, clay- or chalk-pits, isolated trees, paths and so on in reasonable densities must be mentioned. In this respect, the idea of "landscape protection areas" might prove to be especially important for bryophytes.

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## Résumé